

AD\_\_\_\_\_

Award Number: W81XWH-06-1-0723

TITLE:

DNA Hypermethylation Patterns Detected In Serum As A Tool For Early  
Breast Cancer Diagnosis

PRINCIPAL INVESTIGATOR: Jennifer Brooks MSc

CONTRACTING ORGANIZATION: New York University School of Medicine  
New York, NY 10016

REPORT DATE: September 2008

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>				
1. REPORT DATE (DD-MM-YYYY) 01-09-2008		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 1 SEP 2007 - 31 AUG 2008
4. TITLE AND SUBTITLE DNA Hypermethylation Patterns Detected In Serum As A Tool For Early Breast Cancer Diagnosis		5a. CONTRACT NUMBER		
		5b. GRANT NUMBER W81XWH-06-1-0723		
		5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Jennifer Brooks Email: Email:jdb296@nyu.edu		5d. PROJECT NUMBER		
		5e. TASK NUMBER		
		5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  New York University School of Medicine 550 First Avenue New York, NY 10016		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research And Material Command Fort Detrick, Maryland 21702-5012		10. SPONSOR/MONITOR'S ACRONYM(S)		
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Distribution Unlimited				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT The promoter regions of some genes, in particular tumor suppressor genes, are frequently hypermethylated in cancer, but not normal cells. We are conducting a nested case-control study (within the NYUWHS cohort) to assess the potential of serum DNA hypermethylation markers as a tool for early detection of breast cancer. Case-control selection criteria have been designed, the first 200 subject (of 452 subjects in total) selected and cases and their 3 controls matched for age and date of blood donation. DNA has been extracted for these first 200 subjects (50 case-control sets) and stored in aliquots at -20°C until further analysis. DNA methylation analysis requires two basic steps. DNA is chemically modified using sodium bisulfite, creating methylation specific sequence variation that is detectable using quantitative methylation-specific real-time PCR (QMSP). QMSP reactions have been optimized and sensitivity to one genome copy has been attained. Work on the methodological issues surrounding the sodium bisulfite protocol continues. Early detection is an important determinant of breast cancer prognosis and survival. This study is the first to examine aberrant promoter methylation patterns in pre-diagnostic serum samples, taking a step closer to the development of a panel of markers to be incorporated into screening strategies.				
15. SUBJECT TERMS Breast Cancer, Early Diagnosis, DNA Hypermethylation, QMSP				
16. SECURITY CLASSIFICATION OF:		17. LIMITATION OF ABSTRACT  UU	18. NUMBER OF PAGES  23	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U			c. THIS PAGE U

## TABLE OF CONTENTS

<b>INTRODUCTION:</b> .....	<b>4</b>
<b>BODY:</b> .....	<b>4</b>
TRAINING PLAN.....	4
WORK PLAN: .....	5
<b>KEY RESEARCH ACCOMPLISHMENTS:</b> .....	<b>12</b>
<b>REPORTABLE OUTCOMES:</b> .....	<b>13</b>
<b>SUMMARY AND CONCLUSIONS:</b> .....	<b>13</b>
<b>REFERENCES</b> .....	<b>14</b>
<b>APPENDICES</b> .....	<b>15</b>
<b>SUPPORTING DATA:</b> .....	<b>21</b>

## Annual Progress Report – DNA Hypermethylation Patterns in Serum as a Tool for Early Breast Cancer Diagnosis

### Introduction:

The promoter regions of some genes, in particular tumor suppressor genes, are frequently hypermethylated in cancer, but not normal cells. This methylation is thought to be an early event in carcinogenesis. Through necrosis and apoptosis, tumors release genomic DNA into the systemic circulation. Analysis of this DNA found in the serum/plasma of breast cancer cases, allows for the detection of promoter hypermethylation, with results showing good concordance with paired tumor tissue samples. We proposed to assess the potential of serum DNA hypermethylation markers as a tool for early detection of breast cancer. To date, no study has been conducted using serum collected prior to breast cancer diagnosis. Such a study can only be conducted using the resources of a large cohort with access to blood samples collected prospectively in healthy women, such as the NYU Women's Health Study (NYUWHS).

The NYUWHS enrolled 14, 274 women aged 35-65 between the years 1985 and 1991. Serum was collected from each participant and stored for future biochemical analyses.

At the time of the last complete round of follow-up, 1,006 cases of breast cancer had been diagnosed. This project is a nested case-control study within this cohort. Women for whom we have a blood sample collected within the 6 months preceding breast cancer diagnosis (n=113) will form the case group. For each case, controls will be selected and matched for age at, and date of, blood donation. The analysis of the promoter methylation status of a panel of six cancer-related genes (*RASSF1A*, *GSTP1*, *RARβ2*, *ERβ*, *DAPK* and *CDKN2A*) was proposed.

### Body:

Training Plan: The first two tasks listed in the statement of work (coursework and rotations and written preliminary/qualifying exam) were completed prior to the original grant submission. The third task was completed on October 6<sup>th</sup>, 2006 when I successfully defended my dissertation proposal to my PhD advisory committee (Drs Zeleniuch-Jacquotte, Shore, Wirgin, Klein, Liu and Cairns). The discussion generated during the defense lead to a number of modifications to the proposal outline, which were discussed in the previous progress report. Briefly, a second control group consisting of women with a history of benign breast disease (BBD), as indicated by a history of breast biopsy, was added to the proposal. It was also decided that due to the precious nature of this study's samples that the complete analysis (for all 6 genes) should be conducted on 50 sets (1 case and its 3 controls) to allow for a preliminary assessment of how the study is progressing, before analyzing all 113 sets. This is the analysis that has been conducted since that last report.

Also during this last year, I had the opportunity to participate in a National Institute of Environmental Health Science (NIEHS) SNP Workshop at Columbia University. This gave me hands on experience working with SNP and haplotype data and exposure to the large number of database and analytical resources available online. I also took part in this years NYU Cancer Center retreat where researchers come together to discuss their research and attempt to forge collaborations with their fellow cancer center members. In June I attended the DOD Breast

Cancer Conference in Baltimore Maryland. I found the combination of researchers and breast cancer patients and survivors to be one that fostered a climate of discussion and education.

Since the last progress report, I have completed my interim committee meeting (to be completed within the last year of your dissertation research). During this meeting I updated my committee on the research I had completed to date and suggested the steps to be taken to complete my degree. Working on the NYU WHS has also allowed me to be involved in other projects. I published a paper on DNA repair polymorphisms and breast cancer risk (see attached CV) and am currently working on analysis looking at NSAID use and risk.

### Work Plan:

#### Task 1: Case-control Selection

A total of 1,006 invasive breast cancer cases were diagnosed prior to 7/1/03, the start date of our latest complete follow-up. A total of 3,074 women with a history of benign breast disease have also been identified. Cases are women for whom we have a blood sample collected within the 6 months prior to breast cancer diagnosis ( $n=113$ ). For each case, two sets of controls were selected. In the first set, two healthy controls were selected at random from women who were alive and free of any cancer and who had no history of BBD. In the second set, one control subject was randomly selected among healthy cancer-free women with a history of BBD. Controls were matched to cases for age and date of blood donation  $\pm 6$  months.

A series of selection criteria and priorities were created to facilitate the selection of appropriately matched controls. In an ideal match the control's age is within  $\pm 6$  months of the case's age and the date of blood donation in the control is within  $\pm 6$  months of the date of blood donation of the case. To be included in the "healthy" control group, subjects must have been free of benign breast disease at baseline and ANY cancer for the duration of the study to date. Those women in the "Benign Breast Disease" control group needed to be free of ANY cancer for the duration of the study.

The primary objective of this study is to determine whether the promoter methylation status of a panel of genes can be used for the early detection of breast cancer. This makes the cancer free status of the controls the most important selection criterion. To conduct the appropriate comparison between case and control methylation status, needed to meet the study objective, it is important to know that the control did not become a case later on in the study and therefore may have had undiagnosed, early stage breast cancer at the time of blood donation. This is especially true in the case of promoter hypermethylation given that it is believed that these changes occur early on in the development of the tumor.

When an ideal match was not possible, a series of relaxation criteria were established. The first relaxation was to extend the matching for date of blood donation to  $\pm 9$  months while keeping all other criteria the same. If control selection was still not possible then the variation in date of blood donation was increased in 3 month increments up to  $\pm 18$  months. At this point, if a control was still not available, the variation in age was increased in 6 month increments, up to  $\pm 2$  years.

In the first 50 case-control sets, of the 150 controls selected only 7 required the relaxation of selection criteria. For 4 controls the difference in dates of blood donation was extended to  $\pm 9$  months, and for 3 controls to  $\pm 12$  months.

### Task 2: DNA Isolation

DNA was isolated from 1 ml aliquots of serum using the QIAamp DNA Blood Midi Kits (Qiagen, Valencia CA) as described by the manufacturer with a few minor modifications due to the expectation of small amounts of DNA being isolated. These changes have been extensively validated in Dr. Wirgin's laboratory where analysis of NYUWHS DNA has been conducted for the past five years. Samples from each case-control set were isolated in the same batch, on the same day and stored for the same length of time before DNA modification by sodium bisulfite treatment. Isolated DNA was stored in six 45 $\mu$ l aliquots at -80°C to eliminate any unnecessary freeze-thaw. Each aliquot is the amount required for the sodium bisulfite conversion assay and sufficient for the methylation analysis of two genes of interest and the reference gene.

### Task 3: Method Optimization and DNA Methylation Analysis

DNA methylation analysis requires two basic steps. First the DNA must be chemically modified using sodium bisulfite, converting unmethylated cytosines to uracil while leaving methylated cytosines unchanged. This treatment leads to the generation of detectable methylation specific sequence variation. Once treated, DNA is amplified using fluorescence based, quantitative real-time PCR (QMSP) using the AB7300 (Applied Biosystems, Foster City CA). Optimization of the sodium bisulfite treatment method and QMSP analysis has been completed.

#### *Sodium Bisulfite Conversion of DNA:*

In the original proposal sodium bisulfite conversion was to be carried out using the method by Herman et al [1]. However, since the time of the original grant submission a number of kits became available for the sodium bisulfite treatment of DNA. After consultation with those in Dr. Klein's laboratory, in which two different kits had been used, the Qiagen Epitect Bisulfite conversion kit (Qiagen, Valencia CA) was selected. Using the QIAGEN kit increased the speed with which the samples were analyzed. Kits were tested using standards of fully methylated and fully unmethylated DNA (Millipore, Billerica MA). Bisulfite conversion was conducted as described by the manufacturer. Modified DNA not analyzed immediately was stored at -20°C until further use.

Samples from each case-control set were treated in the same batch, on the same day and stored for the same length of time upon conversion. Standards for each PCR plate were also treated in the same batch as the samples for that plate. Usually, samples were analyzed on the same day of sodium bisulfite conversion to eliminate the effects of storage completely.

#### *Analysis of DNA Methylation:*

Bisulfite treated DNA was amplified using QMSP. This method can attain a detection sensitivity of up to 1 in 10,000, compared to a sensitivity of 1 in 1000 for traditional methylation

specific PCR (MSP) [2]. Amplification was conducted using locus and methylation specific primers, flanking a sequence specific, 18-20bp, dual labeled, TaqMan® probe. Increased specificity is gained by the specificity of not only the forward and reverse primers, but the probes as well. Fluorescence was detected using the AB 7300.

Real-time PCR was carried out as described by Eads et al [2]. Briefly, for each assay two sets of primers and probes were used. The first set of primers was designed to recognize the sequence of the methylated, bisulfite treated gene of interest. The second set, for *β-Actin* (*ACTB*), was run in parallel and used as a control to normalize for DNA input. Primer and probe sequences were obtained from previous publications and reported in the first progress report.

Standard curves using fully methylated DNA and probing for the genes of interest and for the control gene (*ACTB*), are included in each plate. This acts as a positive control and allows for the quantification of promoter methylation relative to a fully methylated control. It also controls for sample DNA input. Standard curves are generated from the same stock solution (3.3ng/μl) and can therefore also act as a control for plate-to-plate variability. Dilutions of methylated DNA are run from 10,000 copies (6600 pg DNA/μl) down to 1 genome copy (0.66 pg DNA/μl). Standard curves with high r-squared values and slopes close to -3.33 are the most accurate. Cases and their 3 controls (2 healthy, 1 BBD) are run on the same plate. This ensures that any differences in methylation seen between the 3 groups are not due to plate-to-plate variability or differences in DNA storage time.

To optimize assay efficiency with respect to the limited amount of sample DNA, two target genes were run for each sample on one plate (using one aliquot of isolated DNA). This was run along side the *ACTB* control and allows the same control to be used for both genes. This decreases the amount of sample DNA needed for *ACTB* control reactions overall.

Each assay also included universally unmethylated DNA as a negative methylation control. Unmethylated DNA is included as a negative quality control on each plate to reduce the probability of false positive sample results. Inclusion of this control monitors the specificity of the primers and probes for methylated sodium bisulfite treated sequences as well as the efficiency of the bisulfite treatment reaction itself. Incomplete sodium bisulfite conversion can generate false positive results where unmethylated DNA (i.e. the negative control) is amplified using methylation specific primers. The negative control should only be amplified by *ACTB*, whose primers and probe are not methylation specific. This indiscriminant amplification is what allows it be used to quantify the amount of DNA template in each sample. Several water blanks were also included on each plate.

#### *Amplification Conditions:*

The final composition of the master mix consisted of 1X TaqMan® Universal PCR Master Mix No AmpErase®, 600nM of each primer (forward and reverse) and 200nM MGB probe, with a final reaction volume of 50μl. Amplification conditions were as follows: 10 minutes at 95°C and then 95°C for 15 seconds followed by 60°C for 1 minute, for 50 cycles.

### Gene Selection:

As reported in the previous progress report, the gene panel was modified to include: *RASSF1A*, *HIN-1*, *GSTP1*, *APC*, *p16* and *RAR $\beta$ 2*. Since that last report the panel has changed further. During the extensive troubleshooting that was conducted during the early stages of the study, it was found that the primer/probe set that had been selected for *HIN-1* analysis was not specific to sodium bisulfite treated methylated DNA. This gene was removed from the panel (for the time being) and analysis of *RASSF1A*, *GSTP1*, *RAR $\beta$ 2* and *APC* conducted.

### QMSP Results:

Assays have been completed for the first 50 cases, whose mean age at diagnosis was 53.1 years. Forty percent of the tumors were diagnosed at stage I, 34% at stage 2A, 6% at stage 2B and 2% at stage 3A. Tumor stage was unknown for 9 cases (18%). Methylation assays have shown good sensitivity in the standards (dilutions of fully methylated DNA), able to detect down to one genome copy (**Figure 1**). The standard curves were also the expected slope (-3.33) and had high  $R^2$  values (**Figure 2**). Further, standards were shown to have a high level of reproducibility between plates, as indicated by low inter-plate coefficients of variability for each gene: *RASSF1A* 11%, *GSTP1* 3%, *APC* 2%, *RAR $\beta$ 2* 1%. In contrast however, variability between repeats using sample DNA was very high (i.e. *ACTB* 52%).

Overall, the methylation frequencies of the four analyzed genes are low among all three subject groups (**Table 1**). Further, examination of the frequencies shows that the methylation frequency of these genes does not distinguish between cases, controls with BBD and controls without a history of BBD.

These results raise a number of issues challenges and obstacles that must be addressed. First, the results shown in Table 1 represent all samples that amplified during real-time PCR using methylation specific primers. For some of these samples the amplification crossed the threshold at a high cycle number (i.e. > 45 cycles). This raises the question as to what samples are actually methylated and what amplification is simply an artifact of the PCR procedure. This introduces the potential for the presence of false positives, which may also be due to incomplete sodium bisulfite treatment.

Another issue to be considered at this stage is that of assay sensitivity. The standard curves show that the procedure itself has a very high sensitivity, able to detect down to one genome copy (Figures 1 and 2), however, the assay can only be as sensitive as the sample will allow it. Amplification of *ACTB* in each sample that is not methylation specific is included in each plate to control for DNA input. The average *ACTB* copy number detected for all subjects was only 152 copies. This means that our analytical sensitivity can only reach that of 1 in 152 copies, much less than the technical sensitivity of the procedure itself.

Based on these results, the main issues that need to be addressed are: false positives, reproducibility, small DNA amount and the potential that some characteristic of the samples are making measurements less reliable than that of the standards (as indicated by the reported coefficients of variability). To address these issues, a series of experiments were proposed.



Issue	Method to Address
False Positives	A. Check completeness of Sodium bisulfate treatment B. Confirm results by DNA sequencing
Reproducibility	C. Check the characteristics of the DNA: DNA Fragment Analysis
Sensitivity	D. Nested QMSP

*A: Completeness of Sodium Bisulfite Treatment:*

The question of how well the sodium bisulfite treatment kit works compared to the Herman method of bisulfite treatment is one that was brought up and tested during the first year of the grant and reported in the first progress report. To summarize, it was found that the Herman method resulted in large loss of DNA during the ethanol precipitation step. This greatly reduced the analytical sensitivity of the procedure compared to that seen when using a column, as is done in the Qiagen kit.

The question of whether the kit completely modified the DNA was still an issue. Incomplete modification can lead to false-positive results. Dr. Klein had previously conducted MSP using the Herman method of sodium bisulfite treatment and DNA from various cell lines. To ensure that the Qiagen kit was adequately converting samples, and not producing false positive results - MSP was conducted using the Qiagen sodium bisulfite treatment kit. DNA from four cell lines (MDA-MB-469, MCF-7, HMEC and MCF10A) was analyzed for two genes of interest (GSTP1 and RAR $\beta$ 2). Using this method the results obtained in the lab of Dr. Klein using the Herman method of sodium bisulfite treatment were reproduced indicating that the kit had comparable bisulfite conversion rates (**Figures 3 and 4**).

*B: Confirmation of QMSP by DNA Sequencing:*

To further confirm that the amplification seen with QMSP was due to the presence of methylation and not an artifact of the procedure or incomplete sodium bisulfite conversion, a subset of samples were sequenced (n=31). Because the PCR products of the QMSP reactions are less than 100bp, direct DNA sequencing was not possible and bacterial cloning was required. Samples were selected so that a cross-section of amplification threshold values would be used. This was done in an attempt to determine an appropriate cut-off point to be used for the classification of samples as being methylated.

*Bacterial Cloning Procedure*

DNA samples were sodium bisulfite treated (as described) and MSP was conducted using a final reaction volume of 25 $\mu$ l. This included 1  $\times$  PCR Buffer (Qiagen, Valencia CA), 200 $\mu$ M dNTPs, 60nM of each (forward and reverse) methylation specific primers (Applied Biosystems, Foster City CA) and 1 U Hotstart Taq Polymerase (Qiagen, Valencia CA). Conditions were as follows,

95°C for 15 minutes followed by 45 cycles of 94°C for 20 seconds, 57°C for 30 seconds and 72°C for 30 seconds, followed by a hold at 4°C. Product was visualized by 10% TBE polyacrylamide gel electrophoresis.

The bacterial cloning reaction was carried out using the TOPO<sup>®</sup> TA Cloning Kit for sequencing (Invitrogen, Carlsbad CA). Briefly, the vector ligation reaction was conducted directly after the completion of the MSP program. This reaction included 3µl of fresh PCR product, 1µl of salt solution, 1µl water and 1µl of TOPO<sup>®</sup> vector for a total volume of 6µl. Once combined the reaction was mixed gently and incubated for 5 minutes at room temperature. The reaction was then put on ice or stored at -20°C until use.

Transformation of One Shot<sup>®</sup> TOP10F' Competent Cells (Invitrogen, Carlsbad CA) was carried out using 3µl of ligation reaction and 1µl for the pUC19 control provided. DNA was added directly to a 50µl vial of cells and mixed gently by tapping. Reactions were then incubated on ice for 30 minutes followed by 30 seconds in a 42°C water bath and then back on ice. Using sterile techniques, 250µl of room temperature SOC medium was added to each vial. Reactions were then placed in a rotary shaker incubator on their side and incubated at 37°C for exactly 1 hour while shaking at 225 rpm. Following this, samples were plated on LB Agar plates containing 0.1 mM IPTG, 0.004% galactose in dimethylfluoride and 100µg/µl ampicillin. 100µl of SOC medium was first added to each plate, followed by 80µl from each transformation vial (50µl for the pUC19 transformation control). Plates were then inverted and incubated overnight at 37°C.

The next morning four colonies (1 blue and 3 white) were selected from each plate and placed in culture tubes containing 2ml of LB medium with 100µg/µl ampicillin. Tubes were then put in the rotary shaker incubator overnight and incubated at 37°C while shaking at 225 rpm. After this, cultures were spun down and the medium removed. Plasmid DNA was isolated using the PureLink Quick Plasmid Mini-prep Kit Protocol (Invitrogen, Carlsbad CA) according to the manufactures instructions (**Appendix 1**). Once isolated, DNA was stored at 4°C for immediate use or at -20°C for later use.

### *DNA Sequencing*

Sequencing of isolated plasmid DNA was conducted by capillary gel electrophoresis (CEQ-8000) using a Dye Terminator Cycle Sequencing (DTCS) kit (Beckman Coulter, Fullerton CA). The sequencing reaction was prepared containing 3µl of plasmid DNA, 5pmol of the M13 Reverse plasmid sequencing primer (Invitrogen, Carlsbad CA) and 6µl of DTCS (Beckman Coulter, Fullerton CA) (**Appendix 2**). Reactions were run at 96°C for 20 seconds, 50°C for 20 seconds and 60°C for four minutes, for 40 cycles followed by a hold at 4°C. Samples were then ethanol precipitated and placed in a speed-vac for 15 minutes to dry down. Next, 40µl of sample loading solution (Beckman Coulter, Fullerton CA) was added and samples were capped and allowed to sit for 10 minutes, gently vortexed for 30 seconds and spun down for 10 seconds. Each sample was then transferred to CEQ plates and covered with mineral oil. Plates were run using the shorter LFR-c sequencing program.

Samples that amplified for *RASSF1A* (the gene for which the most samples were amplified with QMSP), were selected for bisulfite sequencing. Results showed that for *RASSF1A*, those samples that amplified were indeed methylated. Sequencing also provided further confirmation that the samples were being completely converted during the sodium bisulfite treatment step. These results suggest that for this gene, any amplification seen with QMSP is due to methylation. It does not however explain why methylation is seen in the control groups.

### C: DNA Quality and Quantity:

The QMSP results showed that while the repeat measures of standards had low variability and good reproducibility, the same was not true for samples. The samples being used for this study are from the NYUWHS which was initiated in the mid-eighties. This means that the serum samples, from which the DNA is obtained, have been stored for approximately 20 years at -80°C. It was hypothesized that one possible reason the samples and standards were behaving differently in the analysis was that the samples had been damaged at some point during their long-term storage.

To test this hypothesis fresh blood samples were collected from 6 healthy individuals. Serum was separated according to the protocol detailed by the NYUWHS [3] and stored at -80°C. DNA was isolated using the Qiagen method and stored in aliquots of 45µl at -80°C until time of analysis. Samples were then analyzed and compared to NYUWHS samples with respect to quantity and quality.

DNA quantity was determined by looking at the copy number obtained for *ACTB* using QMSP. DNA quality was assessed using a PCR based fragment assay as described by van Beers et al [4]. For this experiment isolated DNA (10µl) from freshly collected normal samples and study sample DNA were amplified in a multiplex PCR reaction that included 4 sets of primers specific for fragment sizes of 100, 200, 300 and 400 bps for the *GAPDH* gene. Primer sequences are listed in **Appendix 3**. If samples have a greater proportion of small fragments, the sample is considered to be more fragmented. Fragmentation can disrupt the detection of promoter methylation if it occurs at the primer/probe binding sites.

PCR reactions for this analysis included 1 × PCR Buffer (Qiagen, Valencia CA), 200µM dNTPs, 132nM of each primer (forward and reverse) (Applied Biosystems, Foster City CA) for each fragment size and 1 U Hotstart Taq Polymerase (Qiagen, Valencia CA). Samples were then run for 15 minutes at 95°C and then 1 minute at 94°C, 1 minute at 56°C and 3 minutes at 72°C for 40 cycles followed by 7 minutes at 72°C. Reactions were then visualized on 10% TBE polyacrylamide gel electrophoresis.

*ACTB* was quantified in the fresh samples to determine the number of genome copies present in the samples. After five repeats, *ACTB* was not detectable in two of the samples, later experiments showed that DNA was however present. In those samples that DNA was detectable, there was a high level of variability - as was seen in the NYUWHS samples (**Table 2**). Results of the fragment analysis also showed that though the samples did look somewhat different, the NYUWHS samples were not more fragmented than the fresh DNA samples (**Figure 5**). These results suggest that the issue of sample variability is not due to quality of the

DNA in the NYUWHS samples but perhaps related to the small amount of sample available for analysis.

#### D: Assay Sensitivity:

QMSP can only be as sensitive as the amount of DNA present in the sample. This issue is currently being addressed using a nested QMSP protocol [5]. This procedure involves an amplification step that is not methylation specific, prior to the methylation specific QMSP step. Primers are designed to amplify the gene of interest outside of the area of methylation so that regardless of the methylation status of the gene, it is amplified. This increases the overall amount of DNA that is being input into the QMSP reaction which is carried out in a methylation specific manner as previously described. Optimization of this procedure is currently underway for *RASSF1A*, using primers sequences published by Fackler et al [5]. Once this procedure has been optimized (in the next few weeks) then a selection of NYUWHS samples will be run to test if the sensitivity of the assay can be improved.

#### **Summary:**

QMSP analysis has been completed for four genes (*RASSF1A*, *GSTP1*, *APC* and *RARβ2*) in the first 50 case-control sets. Results have shown that overall the methylation frequency is low and that it is unable to distinguish between cases and their controls. A number of issues were identified during the course of this analysis and steps have been taken to address each of them.

#### Task 4: Statistical Analysis and Manuscript Writing

A review paper outlining the potential of DNA methylation profiles as a tool for breast cancer detection and diagnosis is almost ready for submission.

#### Task 5: Thesis preparation and defense

The writing of the review paper has allowed me to keep on top of the literature for the literature review portion of my thesis. I have also been writing the methods section for my thesis as I have conducted the analysis. Though this writing is still in its early stages, my thesis preparation is on-going and underway.

#### **Key Research Accomplishments:**

- Methylation analysis completed for 4 genes (*RASSF1A*, *GSTP1*, *RARβ2* and *APC*) for the first 50 case-control sets
- Troubleshooting the QMSP Method
- Confirmation of methylation status using bacterial cloning and DNA sequencing
- Sample DNA quality determined to be good
- QM-MSP underway

**Reportable Outcomes:**

See attached CV.

**Grants Received as a result of this Award:**

NYU Cancer Institute Translational Research Pilot Study Grant (Title: Serum Epigenetic Markers for the Early Diagnosis of Breast Cancer, P.I: Dr. Anne Zeleniuch-Jacquotte)  
Funding Period: 1 year (01/07-12/07)  
Amount: \$30,000

Susan G. Komen For The Cure, Basic, Clinical and Translational Research Grant (Title: Serum Epigenetic Markers and the Early Detection of Breast Cancer, P.I: Dr. Anne Zeleniuch-Jacquotte).  
Funding Period: 2 years (07/01/07 – 06/30/09)  
Amount: \$186,724

The Komen grant funds support laboratory supplies and efforts for the mentor of this project and the study data manager. The NYU Pilot Study grant allows for the addition of the BBD control group.

**Summary and Conclusions:**

Over the last year, analysis has been completed for four genes in 50 case-control sets. Analytical issues that have come up during the course of this analysis are being addressed. The results of the analysis show that overall, the methylation frequency for these genes, in these subjects, is low. They also show that promoter methylation is detectable in samples obtained from healthy cancer-free controls. These results are not well understood. Previous studies have included relatively few samples from healthy controls. There remains a need for a greater understanding of normal patterns of DNA methylation in order to understand the changes seen with disease.

## References

1. Herman, J.G., et al., *Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands*. PNAS, 1996. **93**(18): p. 9821-9826.
2. Eads, C.A., et al., *Fields of Aberrant CpG Island Hypermethylation in Barrett's Esophagus and Associated Adenocarcinoma*. Cancer Res, 2000. **60**(18): p. 5021-5026.
3. Toniolo, P., et al., *A prospective study of endogenous estrogens and breast cancer in postmenopausal women*. J Natl Cancer Inst, 1995. **87**(3): p. 190-197.
4. van Beers, E., et al., *A Multiplex PCR Predictor for aCGH success of FFPE samples*. British Journal of Cancer, 2006. **94**: p. 333-337.
5. Fackler, M.J., et al., *Quantitative Multiplex Methylation-Specific PCR Assay for the Detection of Promoter Hypermethylation in Multiple Genes in Breast Cancer*. Cancer Res, 2004. **64**(13): p. 4442-4452.

## Appendices

### Appendix 1:

#### PureLink Quick Plasmid Miniprep Kit Protocol

##### **Before Starting:**

- 1) Add RNase A to **Resuspension Buffer** (R3) as described on label. Mix well. **Store at 4°C.**
- 2) Add 96-100% ethanol to Wash Buffer (W9) and Wash Buffer (W10) according to label. Mix well. **Store at room temperature.**
- 3) If Lysis Buffer (L7) contains salt precipitates, warm buffer in a 37°C water bath until dissolves. **DO NOT** shake the buffer.

##### **Preparing Cell Lysate:**

- 1) Pellet 1-5ml of an overnight culture ( $1-2 \times 10^9$  *E. coli* in LB medium). Thoroughly remove all medium from the cell pellet.
- 2) **Resuspend** the pellet in **250ul Resuspension Buffer** (R3) (with RNase A). No cell clumps should remain.
- 3) **Add 250ul Lysis Buffer** (L7) to cells. **Mix gently by inverting** the capped tube **5 times. DO NOT VORTEX.**
- 4) **Incubate** for 5 minutes at room temperature. **DO NOT** exceed 5 minutes.
- 5) **Add 350ul Precipitation Buffer** (N4). Mix immediately by inverting the tube until homogeneous. For large pellets shake more vigorously. **DO NOT VORTEX.**
- 6) **Centrifuge** at  $\sim 12,000 \times g$  for **10 minutes** at room temperature to clarify the lysate from lysis debris.
- 7) **Load** supernatant from Step 6 onto a Spin Column.

##### **Purification Procedure:**

- 1) **Preheat** an aliquot of TE Buffer (TE) to **65-70°C** for elution (optional).
- 2) Place Spin Column with supernatant into a 2ml Wash Tube.
- 3) **Centrifuge** at  $\sim 12,000 \times g$  for **1 minute**. Discard flow through.

- 4) **Add 500ul Wash Buffer** (W10) (with ethanol) to column. **Incubate** for 1 minute at room temperature. **Centrifuge** at  $\sim 12,000 \times g$  for 1 minute. Discard flow through.
- 5) **Add 700ul Wash Buffer** (W9) (with ethanol) to the column.
- 6) **Centrifuge** at  $\sim 12,000 \times g$  for 1 minute. Discard the flow through.
- 7) **Centrifuge** at  $\sim 12,000 \times g$  for 1 minute to remove residual Wash Buffer. Discard Wash Tube with the flow through.

*Eluting DNA:*

Place Spin Column in clean 1.5ml Recovery Tube.

**Add 75ul** preheated **TE Buffer** to center of column.

**Incubate** for 1 minute at room temperature.

**Centrifuge** at  $\sim 12,000 \times g$  for 2 minutes.

Discard column, purified plasmid DNA is in the recovery tube.

Store DNA at 4°C if using immediately (same day). Otherwise store at -20°C. Avoid freeze thaw so store in aliquots if necessary.

## Appendix 2:

### Dye Terminator Cycle Sequencing Premix Composition

Component	Amount
10X Sequencing Reaction Buffer	200μl
dNTP Mix	100μl
ddUTP Dye Terminator	200μl
ddGTP Dye Terminator	100μl
ddCTP Dye Terminator	200μl
ddATP Dye Terminator	200μl
Polymerase Enzyme	100μl
Total Volume	1100μl

Once prepared, the DTCS premix was aliquoted (to minimize freeze-thaw) and stored at -20°C until use.

## Appendix 3:



GAPDH Primers [4]

100F – gttccaatatgattccaccc

100R – ctcttggaagatggtgatgg

200F – aggtggagcgaggctagc

200R – ttttgcggtggaaatgcct

300F – aggtgagacattcttgctgg

300R – tccactaaccagtcagcgtc

400F – acagtccatgccatcactgc

400R – gcttgacaaagtggcgttg

JENNIFER BROOKS  
 New York University School of Medicine  
 Environmental Medicine - Division of Epidemiology  
 650 1<sup>st</sup> Ave. 5<sup>th</sup> floor  
 W: 212-263-5964 C: 646-496-3090  
 jdb296@nyu.edu

## EDUCATION

- Present      **PhD Candidate:** New York University School of Medicine, Department of Environmental Medicine, Division of Epidemiology  
Thesis Title: Promoter Methylation of Tumor Suppressor Genes Detected in Serum for the Early Detection of Breast Cancer.  
Advisor: Dr. Anne Zeleniuch-Jacquotte
- 2003          **MS:** University of Toronto School of Medicine, Ontario Canada – Department of Nutritional Sciences  
Thesis Title: Phytoestrogens as Modulators of Estrogen Metabolism.  
Advisor: Dr. Lilian U Thompson
- 1998          **BS (Honors, Dean's List) Biomedical Science:** University of Guelph, Ontario Canada

## CERTIFICATES AND TRAINING

- 2004-2008    NYU School of Medicine IBRA HIPAA and Human Subjects Training  
 2007          National Institute of Environmental Health Science Environmental Genome Project: NIEHS SNPs Workshop, Columbia University

## HONORS AND AWARDS

- 2006-2009    Department of Defense Pre-doctoral Training Grant  
 2001-2003    University of Toronto Open Fellowship

## CURRENT FUNDING

Department of Defense Pre-doctoral Training Grant (Title: DNA Hypermethylation Patterns Detected In Serum As A Tool For Early Breast Cancer Diagnosis, P.I: Jennifer Brooks)  
 Funding Period: 3 years (09/06-09/09)  
 Amount: \$90,000

NYU Cancer Institute Translational Research Pilot Study Grant (Title: Serum Epigenetic Markers for the Early Diagnosis of Breast Cancer, P.I: Dr. Anne Zeleniuch-Jacquotte)  
 Funding Period: 1 year (01/07-12/07)

Amount: \$30,000

Susan G. Komen For The Cure, Basic, Clinical and Translational Research Grant (Title: Serum Epigenetic Markers and the Early Detection of Breast Cancer, P.I: Dr. Anne Zeleniuch-Jacquotte)

Funding Period: 2 years (07/01/07-06/30/09)

Amount: \$186,724

## PROFESSIONAL SOCIETIES

- 2005- American Association of Cancer Research (AACR): Molecular Epidemiology Group (MEG) and Women in Cancer Research Group (WICR)  
 2008- International Genetic Epidemiology Society (IGES)

## TEACHING EXPERIENCE

- 2005-2006 Epidemiology, Biostatistics, and Preventive Medicine – NYU School of Medicine  
 Teaching Assistant/Seminar Leader – 30 Medical Students  
 Contact Time: 1hr/week for 6 weeks  
 Prep Time: 6 hours
- 2002 Selected Topics in Food Science – University of Toronto School of Medicine  
 Teaching Assistant – 40 Senior Undergraduate Students  
 Contact Time: 1hr/week for 12 weeks  
 Prep Time: 12 hours

## POSTERS AND PRESENTATIONS

- 2008 Department of Defense Era of Hope Breast Cancer Meeting – Baltimore MD  
 (poster)  
 2007 Annual meeting of the International Collaborative Group on Hormones and Cancer – Gavi, Italy (presentation)  
 2002 Federation of American Societies for Experimental Biology – New Orleans LA  
 (poster)

## PUBLICATIONS

### Original Articles

**Jennifer Brooks**, Wendy Ward, John Hilditch, Jacqui Lewis, Leslie Nickell, Evelyn Wong, Lilian Thompson. Supplementation with flaxseed alters estrogen metabolism in postmenopausal women to a greater extent than does supplementation with an equal amount of soy. Am J Clin Nutr. 2004 Feb;79(2):318-25.

**Jennifer Brooks**, Lilian Thompson. Mammalian lignans and genistein decrease the activities of aromatase and 17 $\beta$ -hydroxysteroid dehydrogenase in MCF-7 cells. *J Steroid Biochem Mol Biol*. 2005 Apr;94(5):461-7.

**Jennifer Brooks**, Roy E Shore, Anne Zeleniuch-Jacquotte, Diane Currie, Yelena Afanasyeva, Karen L Koenig, Alan A Arslan, Paolo Toniolo, Isaac Wirgin. Polymorphisms in *RAD51*, *XRCC2* and *XRCC3* are not related to breast cancer risk. *Cancer Epidemiol Biomarkers Prev*. 2008 Apr;17:1016-19.

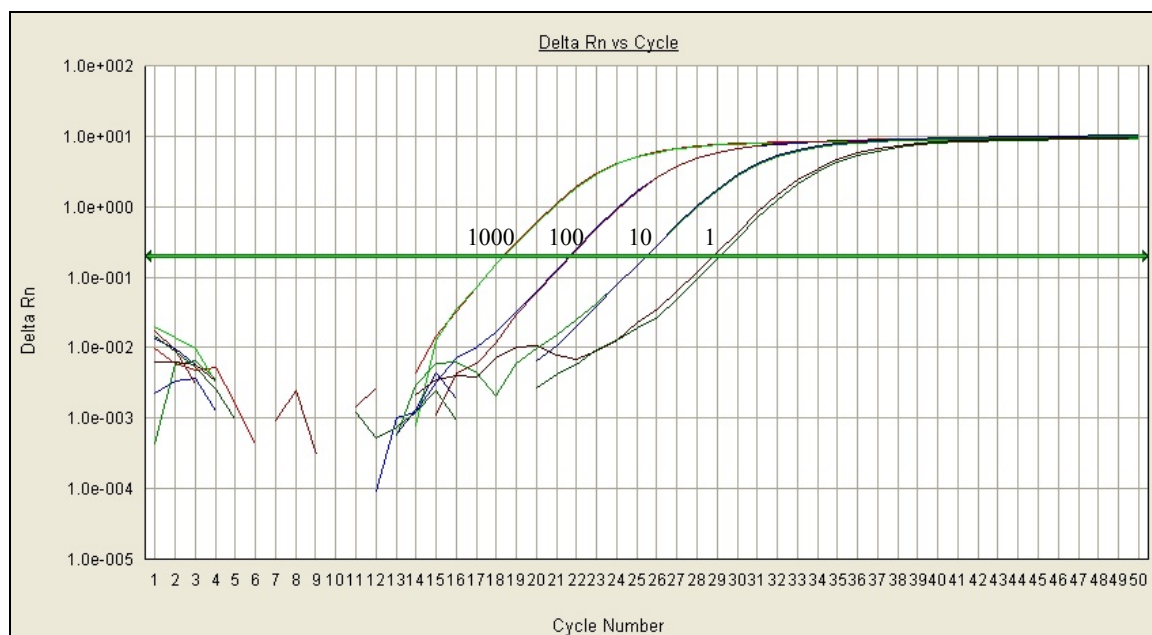
#### Reviews, Chapters and Editorials

Lilian U. Thompson, Jim Chen, Kah Poh Tan, **Jennifer Brooks**, John Hilditch, Paul Gross. Flaxseed, Lignans and Breast Cancer: An Update. Proceedings of the 59<sup>th</sup> Flax Institute of the United States, Fargo, North Dakota, March 21-23: 47-51. 2002.

**Jennifer Brooks**, Anne Zeleniuch-Jacquotte, Paul Cairns. DNA Methylation Analysis for the Early Detection of Breast Cancer (Review): **in preparation**

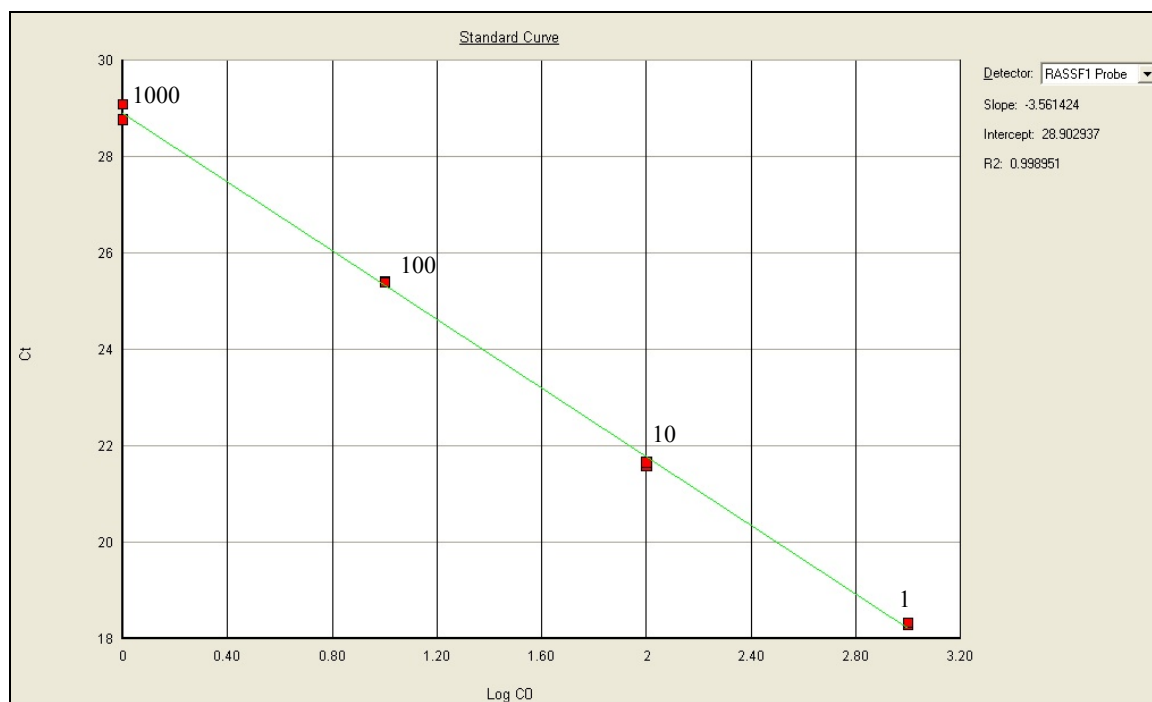
## Supporting Data:

Figure 1: Sensitivity of QMSP Methylation Analysis



Real-Time PCR results for standards of 1000, 100, 10 and 1 genome copies of methylated DNA.

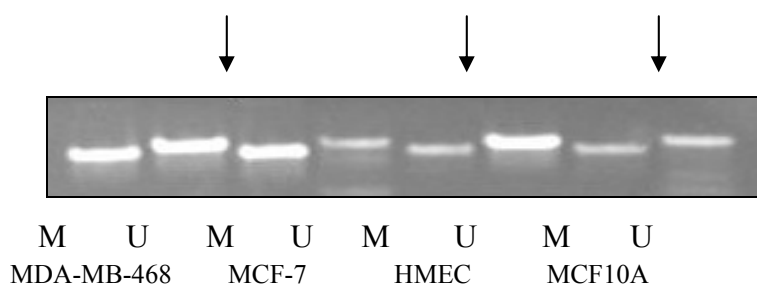
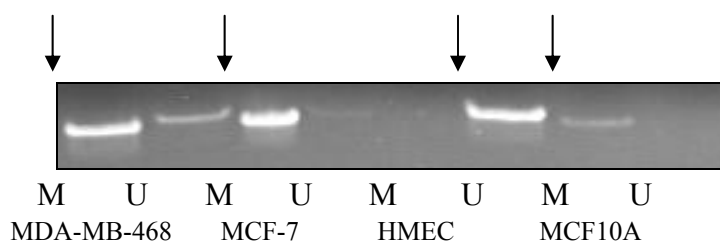
Figure 2: Sample Standard Curve



Standard Curve for 1000, 100, 10 and 1 genome copies of methylated DNA.

Table 1: Methylation Frequencies

Gene	# sets/# subjects analyzed	Case # (%)	Controls with BBD # (%)	Controls w/o BBD # (%)
<i>RASSF1A</i>	47/185	11/47 (23.4)	11/47 (23.4)	17/93 (18.3)
<i>GSTP1</i>	47/185	2/47 (4.3)	5/45 (11.1)	7/93 (7.5)
<i>APC</i>	50/194	1/49 (2.0)	2/47 (4.3)	4/98 (4.1)
<i>RAR<math>\beta</math>2</i>	45/176	3/45 (6.7)	1/43 (2.3)	1/88 (1.1)

Figure 3: Reproduction of MSP Results: *GSTP1*Figure 4: Reproduction of MSP Results: *RAR $\beta$ 2*

**Figures 3 and 4:** Images show the MSP results obtained using the Qiagen sodium bisulfite treatment kit for 4 cell lines and for *GSTP1* (Figure 1) and *RAR $\beta$ 2* (Figure 2). The arrows represent the results obtained in Dr. Klein's lab using the same method except for the Herman method of sodium bisulfite treatment. Dr. Klein's lab did not look at *GSTP1* in MDA-MB-468 so there is no arrow.

Table 2: Average ACTB Copy Number Found in Fresh DNA Samples

Sample	Mean Copy Number	Range
1	ND	NA
2	6.61	0.26-12.78
3	11.43	0.53-21.17
4	ND	NA
5	1.66	0.88-2.49
6	2.34	0.11-5.66

ND: Not detected, NA: Not Applicable

Figure 5: Fragment Analysis of Freshly Collected and NYUWHS DNA

